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Inactivation of Microorganisms by Gamma Irradiation

Bacillus atropheus *spores and* Erwinia herbicola

R.E. Hilsen, B. Kournikakis, B. Ford
DRDC Suffield

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Abstract

The purpose of this study was to establish kill curves detailing the sterilization efficiency of ^{60}Co gamma irradiation on non-pathogenic vegetative and spore preparations. This was done by testing the viability of the microorganisms over a range of gamma irradiation exposures. The procedures used to carry out the work will be used in future sterilization studies of pathogenic microorganisms for later use as antigen reagents. The initial use of non-pathogenic organisms provided a low-risk method for familiarization with the protocol and resolution of any potential problems. Results showed that 4.0 kGy irradiation over a 12 minute period was required for complete inactivation of *Erwinia herbicola.* The inactivation of *Bacillus atropheus* (formerly *Bacillus subtilis var globigii* (BG)) dry powder required 25kGy over 75 minutes and the inactivation of *Bacillus atropheus* in liquid suspension required 35 kGy over 105 minutes. These results showed that vegetative cells and spore preparations can be successfully sterilized by gamma irradiation in a short amount of time. These results will be used as starting points to establish kill curves for Risk Group 3 organisms such as *Bacillus anthracis* and *Yersinia pestis.*

Résumé

L'objectif de cette étude consistait à établir des courbes de neutralisation détaillant l'efficacité de la stérilisation par irradiation gamma ^{60}Co sur des préparations végétatives non pathogéniques et de spores. Ceci à été réalisé en testant la viabilité des microorganismes dans toute une gamme d'irradiations par expositions. Les procédures utilisées pour effectuer ces travaux serviront plus tard à l'étude de la stérilisation des microorganismes pathogéniques qui seront utilisés plus tard comme réactifs d'antigènes. L'utilisation initiale d'organismes non pathogéniques est une méthode à faible risque permettant la familiarisation avec le protocole et la résolution des problèmes potentiels. Les résultats indiquent qu'il faut une irradiation de 4.0 kGy durant une période de 12 minutes pour inactiver complètement *Erwinia herbicola.* L'inactivation du *Bacillus atropheus* (autrefois appelé *Bacillus subtilis var globigii* (BG) en poudre sèche exigeait 25 kGy durant une période de 75 minutes et l'inactivation du *Bacillus atropheus* en suspension liquide exigeait 35 kGy durant une période de 105 minutes. Ces résultats indiquaient qu'il est possible de stériliser les préparations de cellules végétatives et de spores par des irradiations gamma en peu de temps. Ces résultats seront utilisés comme points de départ pour établir les courbes de neutralisation d'organismes du Groupe du risque 3 tels que *Bacillus anthracis* et *Yersinia pestis.*

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Executive summary

Introduction

Detection assays in the laboratory often employ inactivated preparations of vegetative bacteria or spores as sources of antigens. Chemical (e.g. formaldehyde) and thermal (e.g. autoclave) inactivation can be used in the preparation of antigens but the antigen structure itself can be adversely affected by the inactivation process. The use of gamma irradiation provides better preservation of the antigenic structure while still providing a reliable method of inactivation. An MDS Nordion Gammacell 220 Excel with a ^{60}Co source was used for this study.

The purpose of this study was to establish kill curves detailing the sterilization efficiency of ^{60}Co gamma irradiation on non-pathogenic vegetative and spore preparations. This was done by testing the viability of the microorganisms over a range of gamma irradiation exposures. The procedures used to carry out the work will be used in future in the sterilization of pathogenic microorganisms for use as antigen reagents. The initial use of non-pathogenic organisms provided a low-risk method for familiarization with the protocol and its' refinement if problems were identified.

Results

Results showed that 4.0 kGy irradiation over a 12 minute period was required for complete inactivation of *Erwinia herbicola.* The inactivation of *Bacillus atropheus* (formerly *Bacillus subtilis var globigii* (BG)) dry powder required 25 kGy over 75 minutes and the inactivation of *Bacillus atropheus* in liquid suspension required 35 kGy over 105 minutes. These results showed that vegetative cells and spore preparations can be successfully sterilized by gamma irradiation in a short amount of time.

Significance

The gamma inactivation data in this study will be used to define the initial exposure range for further study of gamma irradiation bacterial Risk Group 3 agents (e.g. *Bacillus anthracis* and *Yersinia pestis* held by DRDC Suffield). Organisms inactivated by gamma irradiation could then be safely used as killed antigen preparations. This provides an important research tool as this type of material is not available from known commercial sources.

Future Considerations

Since genetic repair and metabolic recovery of microorganisms after irradiation has been documented [3], sterility counts should be repeated again days later to check for any surviving organisms. It would also be beneficial for understanding the efficiency of radiation sterilization to determine the relationship between the dose of gamma irradiation delivered and the amount of organism being irradiated.

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Sommaire

Introduction

Les biotests de détection en laboratoire emploient souvent des préparations inactivées de bactéries végétatives ou de spores comme sources d'antigènes. L'inactivation chimique (ex : formaldéhyde) et thermique (ex : autoclave) peut être utilisée dans la préparation des antigènes mais la structure d'antigène elle-même peut être affectée de façon négative par le processus d'inactivation. L'utilisation d'irradiation gamma préserve mieux la structure antigénique tout en étant une méthode fiable d'inactivation. On a utilisé *MDS Nordion Gammacell 220 Excel* avec une source de ^{60}Co pour cette étude.

L'objectif de cette étude consistait à établir des courbes de neutralisation détaillant l'efficacité de la stérilisation par irradiation gamma ^{60}Co sur des préparations végétatives non pathogéniques et de spores. Ceci à été réalisé en testant la viabilité des microorganismes dans toute une gamme d'irradiations par expositions. Les procédures utilisées pour effectuer ces travaux seront utilisées dans le futur pour l'étude de la stérilisation des microorganismes pathogéniques devant être utilisés plus tard comme réactifs d'antigènes. L'utilisation initiale d'organismes non pathogéniques est une méthode à faible risque permettant la familiarisation avec le protocole et ses raffinements quand des problèmes sont identifiés.

Résultats

Les résultats indiquent qu'il faut une irradiation de 4.0 kGy durant une période de 12 minutes pour inactiver complètement *Erwinia herbicola.* L'inactivation du *Bacillus atropheus* (autrefois appelé *Bacillus subtilis var globigii* (BG) en poudre sèche exigeait 25 kGy durant une période de 75 minutes et l'inactivation du *Bacillus atropheus* en suspension liquide exigeait 35 kGy durant une période de 105 minutes. Ces résultats indiquaient qu'il est possible de stériliser les préparations de cellules végétatives et de spores par des irradiations gamma en peu de temps.

La portée des résultats

Les données d'inactivation gamma de cette étude seront utilisées pour définir la gamme d'expositions initiales pour les études ultérieures sur l'irradiation gamma des agents bactériens du Groupe de risque 3 (ex : *Bacillus anthracis* et *Yersinia pestis* que possède RDDC Suffield). Des organismes inactivés par irradiation gamma pourraient ensuite être utilisés de manière sécuritaire comme préparations d'antigènes morts. Ceci est un outil de recherche très important puisque ce type de matériau n'est pas disponible chez les sources commerciales.

Éléments à prendre en considérations pour le futur

La réparation génétique et le rétablissement métabolique des microorganismes après irradiation ayant été documentés [3], les comptages de stérilité devraient être répétés de nouveau quelques jours plus tard pour vérifier si des organismes ont survécu. Ceci serait aussi bénéfique pour comprendre l'efficacité de la stérilisation par radiation et déterminer le rapport entre la dose d'irradiation gamma donnée et la quantité d'organismes irradiés.

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1. Introduction

Sterilization of biological products by radiation is not a new technology, but it is undergoing a revivial. Irradiation of biological products by gamma rays has several advantages, including independence of packaging, excellent reproducibility, and the ability to preserve biological function such as antigencity, protein acitivty [1], or even food value [2]. Gamma irradiation kills microogranisms by direct modification (e.g. crosslinking) of proteins and nucleic acids, and by free-radical effects in free water. The mechanisms of radiation effects on biomaterials (or packaging materials) could potentially compromise the structural integrity of the packaging causing leakage or breakage, thus the balance of achieving sterilization and preserving the residual structure or function of the irradiated materials must be obtained. Like other sterilization modes, chemical or physical, irradiation of various types of preparation requires validation and standardization. Ideally, the functional properties of the preparation can be preserved, while achieving sterilization to the point where no infectious or contaminating live organisms remain.

2. Materials and Methods

Three different experimental preparations covering both vegetative and spore forming organisms were exposed to gamma irradiation in triplicate:

2.1 Vegetative Cell Preparation

Overnight cultures of *Erwinia herbicola*, strain number 8L5 from the Brooks Horticultural Research Station (Brooks, AB) (2), with a stock concentration of 8.08×10^9 cfu/mL were grown in Trypticase Soy Broth (TSB), lot number 0038002 from Becton Dickinson (Cockeyville, MD). A stock culture of 125mL of fresh TSB was inoculated with 250 µl of *Erwinia herbicola* grown previously in TSB. *Erwinia* stock was grown overnight at 37°C with shaking at 125 rpm. Stock culture was then aliquotted into twelve 15 mL tubes with 10 mL of stock into each tube.

2.2 Spore Preparations

Dry Spore Preparation:

Dry *Bacillus atropheus* (formerly *Bacillus subtilis var globigii* (BG)) stock powder was weighed out into ten 15 mL tubes. A total of 0.01 g was added to each tube. The spore stock (donated by the US Department of Defense, Dugway Proving Ground, Utah), is held in dry powder form with a stock concentration of ~1×10^{11} cfu/gm.

Spore Preparation in Liquid Media :

0.01 g of the same dry BG stock was added to 110mL sterile double-distilled water in a 250 mL flask. The preparation was mixed vigorously and aliquotted into ten 15 mL tubes with a total of 10 mL of this stock aliquotted into each tube.

2.3 Gamma Irradiation

Bacterial samples were gamma irradiated in 15 mL polypropylene centrifuge tubes (Fisher Scientific, Ottawa, ON). Each tube was vortexed (with the exception of the dry BG stock powder), prior to being placed into a new unlabelled metal paint can (~ 10.7cm w x 12.4 cm h). The paint can was then loaded into the sample chamber at the top of the Gammacell unit. The irradiation timer was set for the appropriate exposure time. Gamma irradiation was carried out with the Gammacell 220 Excel ^{60}Co (manufactured by MDS Nordion) as per the "Standard Operating Procedures Gammacell 220 EXCEL".

The spore preparation samples received radiation doses from 0 to 50 kGy in increments of 4.6 kGy (15 minute intervals), whereas the vegetative cell preparation received radiation doses from 1 to 4 kGy in 0.30 kGy increments (one minute intervals). The gamma source dose rate during the course of this study was 18.21 kGy per hour or 0.303 kGy/min. ^{60}Co has a half life of 5.24 years so the time required to achieve a desired level of irradiation will increase with time. A chart is provided (Appendix A) for future dose calculations

2.4 Viability Assays

Stock samples and gamma irradiated samples were 10-fold serially diluted in sterile double-distilled water. Total viable counts for the appropriate dilutions of either stock or gamma irradiated samples were carried out by spread plating 250 uL of each stock or sample onto Nutrient Agar plates (Nutrient Agar powder, lot number 213000 from Becton Dickinson, Sparks, Maryland), using the Autoplate 4000 from Spiral Biotech. All stocks and samples were plated in duplicate and incubated overnight at 37°C. Plates were counted using the CASBA 4 with Colony Imagine Analysis (Spiral, Biotech) and the data exported to Microsoft Excel 2003 and Sigma Plot 8.0 for graphical analysis.

3. Results

3.1 Inactivation of Vegetative Cells

A total of twelve 10 mL samples containing *Erwinia herbicola* in TBS were gamma irradiated at one minute increments. The initial stock concentration of 3.79×10^8 cfu/mL, showed a nine log reduction at complete inactivation with 4.0 kGy. It took 12 minutes to deliver this dose to the last sample, number 12.

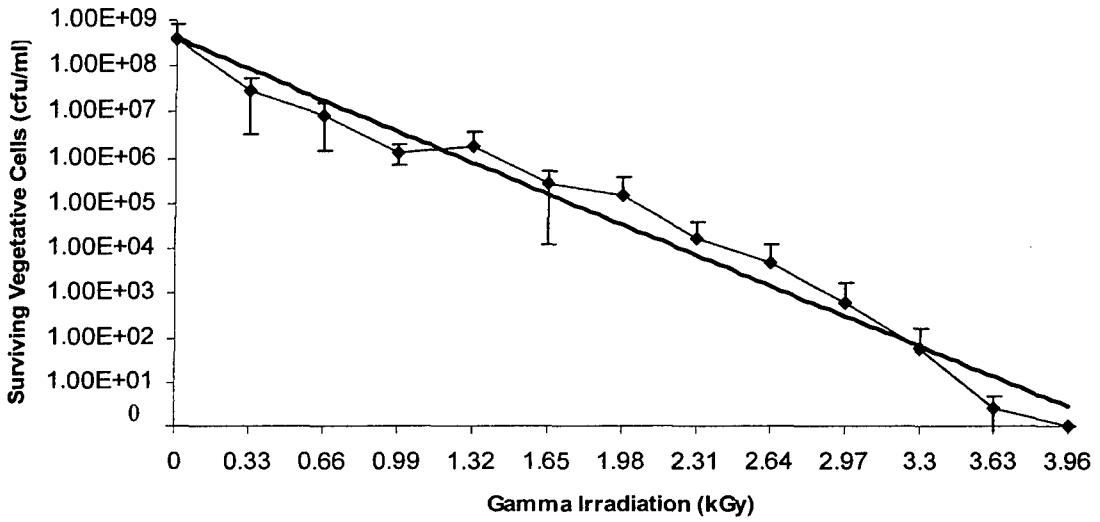

Figure 1: Gamma Irradiation Kill Curve for Erwinia herbicola. A total of 12 Erwinia herbicola culture samples, each in 10 mL TSB, were gamma irradiated at one minute increments. Complete inactivation is seen with 4.0 kGy at 12 minutes. This corresponds to sample number 12. Results are the average of three experiments ± 1 standard deviation.

3.2 Inactivation of Dry Spores

A total of ten samples containing 0.01g of *Bacillus atropheus* dry powdered spores were gamma irradiated at 15 minute increments. The initial stock concentration of 1.06×10^7 cfu/mL showed a seven log reduction at complete inactivation with 25 kGy. It took 75 minutes to deliver this dose to sample number five.

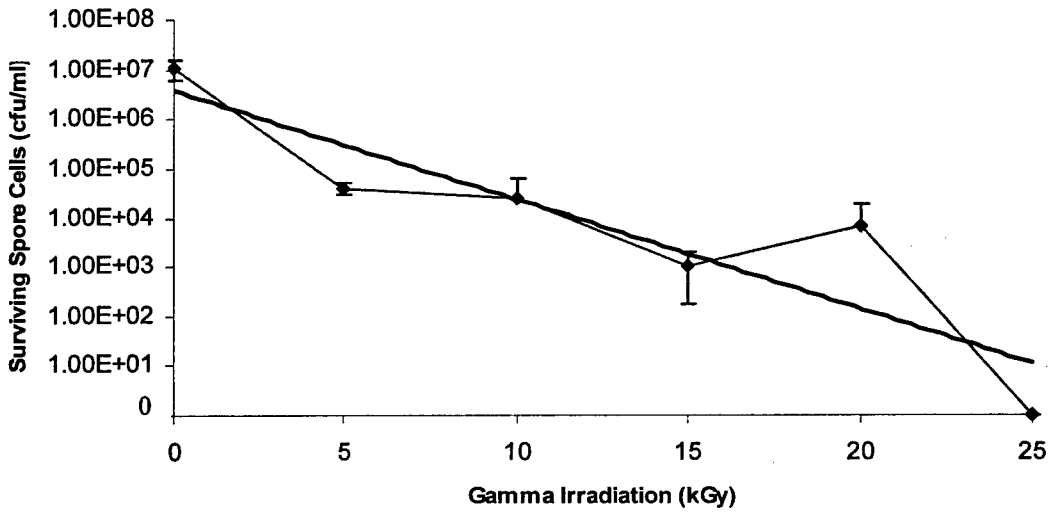

Figure 2: Gamma Irradiation Kill Curve for Bacillus atropheus powder. Ten 0.01 g Bacillus atropheus samples were gamma irradiated at 15 minute increments. Complete inactivation is seen with 25 kGy at 75 minutes. This corresponded to sample number five. Results are the average of three experiments ± 1 standard deviation.

3.3 Inactivation of Spores In Suspension

A total of ten 10 mL samples containing 0.01g of *Bacillus atropheus* in water were gamma irradiated at 15 minute increments. The initial stock concentration of 8.25 x 10^6 cfu/mL showed a seven log reduction at complete inactivation with 35 kGy. It took 105 minutes to deliver this dose to sample number seven.

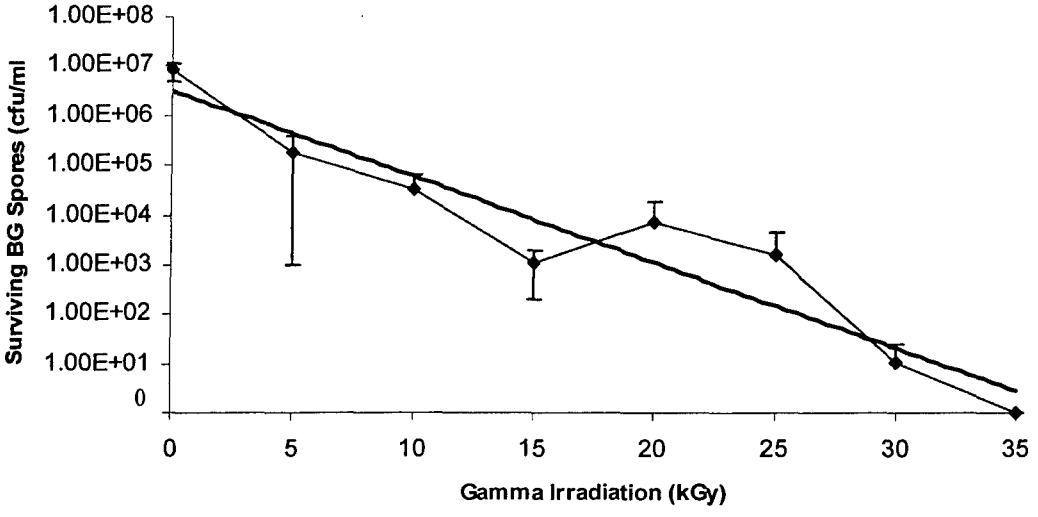

Figure 3: Gamma Irradiation Kill Curve for Bacillus atropheus in suspension. Ten 10 mL samples of 0.01 g Bacillus atropheus in water were gamma irradiated at 15 minute increments. Complete inactivation is seen with 35 kGy at 105 minutes. This corresponded to sample number seven. Results are the average of three experiments ± 1 standard deviation.

3.4 Sample Tube Suitability

Although some of the 15 mL polypropylene tubes used for this study were exposed to gamma irradiation for over an hour, the only result of this gamma exposure was some slight yellowing. None of the sample tubes showed any visible damage due to the exposure to gamma irradiation. The appearance of the tubes exposed for the maximum amount of time indicated that the polypropylene tubes can be safely used for gamma irradiation of both wet and dry samples for at least the 150 minutes maximum dose used during this study.

4. Discussion

Detection assays in the laboratory often employ inactivated preparations of vegetative bacteria or spores as sources of antigens. Chemical (e.g. formaldehyde) and thermal (e.g. autoclave) inactivation can be used in the preparation of antigens but the antigen structure itself can be adversely affected by the inactivation process. The use of gamma irradiation provides better preservation of the antigenic structure while still providing a reliable method of inactivation. An MDS Nordion Gammacell 220 Excel with a ^{60}Co source was used for this study.

A study carried out at DSTL Porton Down [4] used gamma irradiation to inactivate *Bacillus anthracis* (Sterne and Ames strains) vegetative cells and spores. This study provides us with a preliminary range of radiation exposures. DSTL Porton Down determined that vegetative cells required 2.85 kGy and spores 41.5 kGy to achieve a reliable and reproducible 100% inactivation of the organisms. The irradiation source used at Porton Down delivered a dose of 0.9 kGy per hour so that an exposure time of 50 hours was required to kill the spores. The DRDC Suffield Gammacell source is more powerful, providing a 19.4 kGy per hour exposure. Our data suggested that 100% inactivation of spores could be achieved in 105 minutes.

Inactivation of either vegetative or spore forming organisms by gamma irradiation is shown to be a convenient and reproducible technique. Organism preparations, either in suspension or in the dry state, are successfully inactivated by gamma irradiation. Vegetative cells are very sensitive to gamma radiation whereas spore forming bacteria require a much higher dose of gamma irradiation. However, the magnitude of this inactivation depends on the type of organism, the irradiation dose and physical state of organism.

The gamma inactivation data in this study will be used to define the initial exposure range for further study of gamma irradiation of bacterial Risk Group 3 agents (e.g. *Bacillus anthracis* and *Yersinia pestis*) held by DRDC Suffield. The gamma inactivated organisms could then be safely used as killed antigen preparations. This provides an important research tool since this type of material is not available from known commercial sources.

5. References

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3. Clavero M.R., Monk J.D., Beuchat, L.R., Doyle, M.P., Brackett, R.E. Inactivation of Escherichia coli O157:H7, salmonellae, and Campylobacter jejuni in raw ground beef by gamma irradiation. *Appl Environ Microbiol.* 1994 Jun;60(6):2069-75.

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ANNEX A – Gammacell 220 Decay Graph

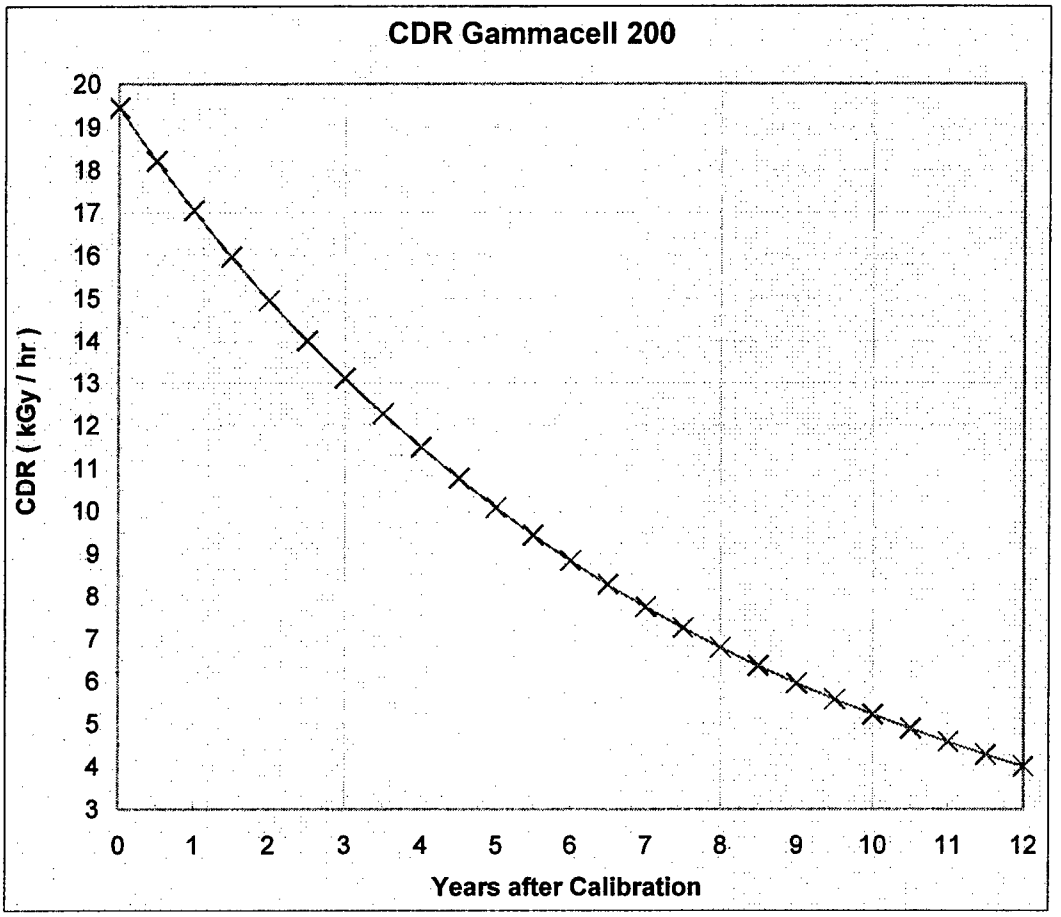

Figure A1: Gammacell 220 Decay Graph. The above graph shows the decay curve for the Gammacell 220. Date at time 0 is 12 March 2004. (Received from Dr. Barry Ford).

ANNEX B – Gammacell 220 Decay Chart

Years from Calibration	Date	CDR kGy/hr	Dose in 10 minutes kGy	Kill Curve Correction Factor	Kill Time Correction Factor
0.00	12-Mar-04	19.45	3.24	1.00	1.00
0.50	12-Sep-05	18.21	3.04	0.94	1.07
1.00	12-Mar-05	17.06	2.84	0.88	1.14
1.50	12-Sep-06	15.97	2.66	0.82	1.22
2.00	12-Mar-06	14.95	2.49	0.77	1.30
2.50	12-Sep-07	14.00	2.33	0.72	1.39
3.00	12-Mar-07	13.11	2.19	0.67	1.48
3.50	12-Sep-08	12.28	2.05	0.63	1.58
4.00	12-Mar-08	11.50	1.92	0.59	1.69
4.50	12-Sep-09	10.76	1.79	0.55	1.81
5.00	12-Mar-09	10.08	1.68	0.52	1.93
5.50	12-Sep-10	9.44	1.57	0.49	2.06
6.00	12-Mar-10	8.84	1.47	0.45	2.20
6.50	12-Sep-11	8.27	1.38	0.43	2.35
7.00	12-Mar-11	7.75	1.29	0.40	2.51
7.50	12-Sep-12	7.26	1.21	0.37	2.68
8.00	12-Mar-12	6.79	1.13	0.35	2.86
8.50	12-Sep-13	6.36	1.06	0.33	3.06
9.00	12-Mar-13	5.96	0.99	0.31	3.27
9.50	12-Sep-14	5.58	0.93	0.29	3.49
10.00	12-Mar-14	5.22	0.87	0.27	3.72
10.50	12-Sep-15	4.89	0.82	0.25	3.98
11.00	12-Mar-15	4.58	0.76	0.24	4.25
11.50	12-Sep-16	4.29	0.71	0.22	4.54
12.00	12-Mar-16	4.01	0.67	0.21	4.84
12.50	12-Sep-17	3.76	0.63	0.19	5.17

Table 1: Gammacell 220 Decay Chart. The above chart indicates gamma irradiation dose rate change over time. Due to ^{60}Co having a half life of 5.24 years, the time required to achieve the initial central dose rate (kGy/hr) at 0.00 years from calibration will increase, as shown by the "Kill Time Correction Factor". (Received from Dr. Barry Ford).

List of symbols/abbreviations/acronyms/initialisms

BG	*Bacillus atropheus* (formerly *Bacillus subtilis var globigii*)
BSL	Biological Safety Level
^{60}Co	cobalt 60
cfu/ml	colony forming units per millilitre
°C	Degrees Celsius
g	grams
h	height
kGy	kiloGrays
kGy/min	kiloGrays per minute
ul	microlitres
ml	millilitre
min	minute
rpm	revolutions per minute
TSB	Tripticase Soy Broth
w	width

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The purpose of this study was to establish kill curves detailing the sterilization efficiency of ^{60}Co gamma irradiation on non-pathogenic vegetative and spore preparations. This was done by testing the viability of the microorganisms over a range of gamma irradiation exposures. The procedures used to carry out the work will be used in future sterilization studies of pathogenic microorganisms for later use as antigen reagents. The initial use of non-pathogenic organisms provided a low-risk method for familiarization with the protocol and resolution of any potential problems. Results showed that 4.0 kGy irradiation over a 12 minute period was required for complete inactivation of *Erwinia herbicola.* The inactivation of *Bacillus atropheus* (formerly *Bacillus subtilis var globigii* (BG)) dry powder required 25kGy over 75 minutes and the inactivation of *Bacillus atropheus* in liquid suspension required 35 kGy over 105 minutes. These results showed that vegetative cells and spore preparations can be successfully sterilized by gamma irradiation in a short amount of time. These results will be used as starting points to establish kill curves for Risk Group 3 organisms such as *Bacillus anthracis* and *Yersinia pestis.*

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Cobalt-60, gamma irradiation, kill curve, Bacillus atropheus, Erwinia herbicola